Identifying Metabolic Phenotype Switches in Cancer Cells Using the Agilent Seahorse XF Analyzer in an Hypoxic Environment

Application Note

Introduction

Decreased oxygen levels, or hypoxia, and hypoxic-mediated signaling play an important role in the pathology of diseases including stroke, ischemic heart disease, renal fibrosis, and cancer[1]. It has been noted in the literature that many hypoxia-regulated genes control substrate supply for glucose transporters as well as other cellular metabolic pathways. For example, cancer cells reprogram their metabolic phenotype to a preferential reliance on glycolysis for ATP generation. This switch is termed the Warburg effect; in many types of cancer cells, substrate preference is altered to conform to the pressures and demands caused by changes in the cellular environment, including oxygen levels[2,3].

Agilent Seahorse XF technology has been adapted for use in reduced O_2 environments (hypoxia chambers) to assess alterations in metabolic function in hypoxia-exposed cells. The use of a hypoxia chamber combined with Agilent Seahorse XF Analyzer software (XF Hypoxia Rate Calculator or Hypoxia Mode for Wave) has been shown to yield accurate measurements of cellular respiration and glycolysis at low O_2 concentrations.

This Technical Overview demonstrates the metabolic differences in MCF-7 human breast cancer cells cultured and assayed at either ambient or 5 % oxygen levels, by measuring mitochondrial respiration and glycolytic activity using the Agilent Seahorse XF Analyzer and XF Hypoxia Rate Calculator.

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What do we mean by hypoxia

In vivo, cells typically encounter environments with 3–5 % O_2. Most *in vitro* cultures however, are maintained under atmospheric oxygen (~21 % O_2) levels. Therefore, culturing and assaying cells at low oxygen levels, or hypoxia, will more closely simulate *in vivo* conditions, and improve model systems.

Why recalculate the oxygen consumption rate (OCR)

XF software and Wave employ an algorithm that corrects for oxygen absorbed by the cell culture plates that is diffused into the culture medium during the assay[1]. When an XF assay is conducted under low oxygen conditions, recalculating with the XF Hypoxia Rate Calculator or Hypoxia Mode for Wave provides accurate OCR values. Failure to recalculate OCR with the oxygen set-point used in the assay, and the hypoxia well group zero oxygen reference, will result in inaccurately high rates of oxygen consumption.

Materials and Methods

The hypoxia assay workflow (Figure 1) provides an overview of the XF Hypoxia Assay. For detailed material and methods, refer to *Protocol: Conducting an XF Assay in a Hypoxia Chamber*[4].

Cell culture

MCF-7 human breast cancer cells were either cultured at ambient or 5 % O_2, using a separate low O_2 incubator. Twenty-four hours prior to the experiments, the cells were counted and seeded at a predetermined optimal density in XF96 Cell Culture Microplates[4].

Loading the XF assay cartridge

Concentrations and volumes of assay reagents were optimized for the assay conditions. A 1.0 M sodium sulfite solution, prepared in XF Calibrant, was added to ports A, B, and C in column 12 in accordance with the Protocol[4].

Medium exchange

Refer to the Protocol[4] to equilibrate the XF Assay Medium to assay conditions.

XF Assay (under hypoxia)

Agilent Seahorse XF Glycolysis Stress Test and XF Cell Mito Stress Test assays (p/n 102194-100 and 101706-100, respectively) were conducted according to manufacturer's instructions.

Data analysis

Following XF assay completion, XF Software generates a data file in Microsoft Excel. This file requires recalculation to account for the reduced oxygen level. In the experiments described, an XF96 Analyzer was used in conjunction with the XF Hypoxia Rate Calculator, a separate recalculation software program installed alongside the XF96 software (Note: XFe Analyzers have the recalculation tool built into Wave).

For these experiments, the XF Excel files were recalculated using the XF Hypoxia Rate Calculator; the assay %O_2 was adjusted to 5 % to reflect the experimental conditions. The recalculated values were then used for data analysis and interpretation.

Results interpretation

The XF Hypoxia Rate Calculator software program was used to analyze the XF data. MCF-7 cells demonstrate the use of the XF Analyzer to examine metabolic changes under hypoxic conditions.

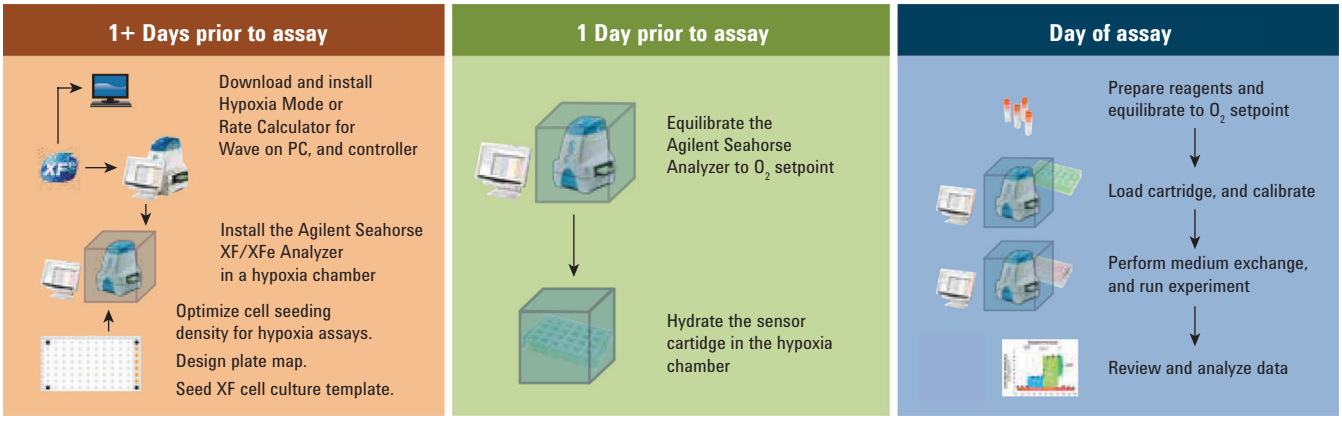

Figure 1. Hypoxia assay workflow.

Hypoxia Alters the Metabolic Phenotype of Cancer Cells

To examine the effects of hypoxia on global metabolic function in MCF-7 cells, basal OCR and ECAR rates were compared. Cells were cultured for 24 hours, either at ambient or 5 % O_2, and assayed under their respective O_2 conditions. Baseline OCR and ECAR data from these experiments were combined to generate an XF Energy Map to visualize the differences in metabolic function (Figure 2). These data indicate that MCF-7 cells exhibit a characteristic Warburg shift in metabolism, in that the decrease in mitochondrial oxygen consumption is observed to be compensated by an increase in glycolysis[5].

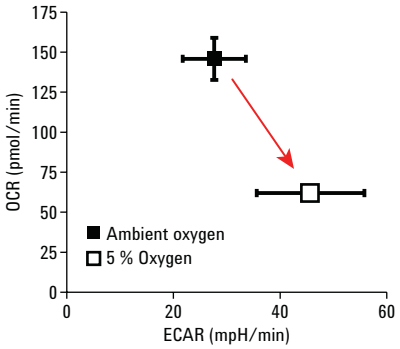

Figure 2. MCF-7 cells exhibit a characteristic Warburg shift following exposure to 5 % O_2. MCF-7 cells were cultured and assayed in the indicated O_2 environment. Baseline OCR and ECAR from each experiment were plotted as an XF Energy Map. Data shown are means ± sd. n ≥ 7 per treatment group.

Stimulation of Glycolytic Rate Following Hypoxia Exposure

MCF-7 cells were exposed to 5 % O_2 for 16 hours to assess the effect of hypoxia on the glycolytic rate and capacity. Glycolytic response was determined using the XF Glycolysis Stress Test, with cells assayed under the same oxygen culturing conditions. Figure 3 shows MCF-7 cells cultured in low substrate media (that is, media without added glucose, glutamine, or pyruvate) had a significantly increased response to glucose injection when cultured in hypoxic conditions as compared to normoxia. Differences in cell growth were not observed during the assays. These data are consistent with those illustrated in Figure 2.

To test the stressed glycolytic rate of hypoxia and normoxia-exposed cells, oligomycin was injected during the XF Glycolysis Stress Test to inhibit mitochondrial ATP production. A typical response includes an increase in ECAR to compensate for the ATP loss. However, a difference was not observed in the oligomycin-stimulated ECAR, between hypoxia and normoxia-exposed cells.

Lastly, 2-deoxy-D-glucose (2-DG) was injected to determine the specificity of the glycolytic ECAR measurement. In both treatment groups, ECAR was significantly reduced reminiscent of levels associated with glucose-free medium. Collectively, these observations indicate that the increase in glucose- and oligomycin-stimulated ECAR can be attributed to glucose metabolism.

Inducers of HIF Stabilization Promote a Similar Increase in ECAR

Hypoxia inducible factor 1 (HIF1) is a key regulator in a hypoxia-mediated response and its stabilization a classic hallmark of hypoxia. HIF-1, composed of HIF-1α and HIF-1β, can activate a broad range of pathways, including angiogenesis, apoptosis, and glucose metabolism[1,6].

Demonstrating that the effect of hypoxia observed using the XF Glycolysis Stress Test (Figure 3) was due to HIF-1α stabilization and not limited oxygen availability to the mitochondria; known HIF-1α inducers were tested to observe the effects on ECAR in cells cultured in normoxia.

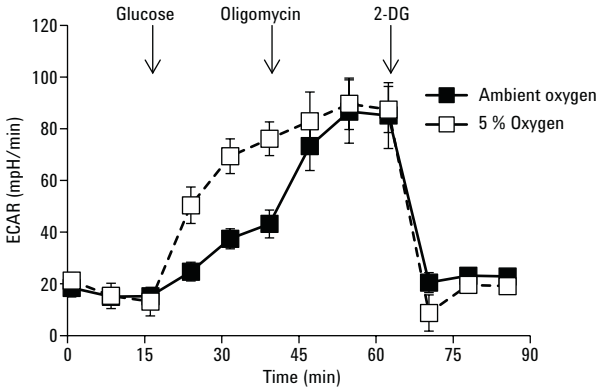

Figure 3. The Agilent Seahorse XF Glycolysis Stress Test demonstrates an increase in glycolytic response following exposure to hypoxia in MCF-7 cells. Cells were cultured at 5 % oxygen, as described. The Agilent Seahorse XF Glycolysis Stress Test, composed of serial injections of glucose (10 mM), oligomycin (1 µM), and 2-DG (100 mM), was performed at the indicated O_2 levels. Data shown are means ± sd. n ≥ 7 per treatment group.

Figure 4 shows that glycolysis was induced in normoxia-exposed cells treated with $CoCl_2$ without increasing oligomycin-induced ECAR. This observation mirrors the effect observed in Figure 3 with hypoxia-exposed cells. These data suggest that HIF stabilization is responsible for the increased glycolysis observed.

Exposure to Hypoxia Decreases Mitochondrial Function

The majority of human cell oxygen consumption is via cytochrome C oxidase in the electron transport chain. It has been observed that the turnover rate of this enzyme is not affected by oxygen availability until the cellular concentration has been reduced to ~0.1 %[7]. However, a reversible decrease in oxygen consumption during hypoxia exposure has been reported, likely owing to the increase glycolytic rate described above.

The XF Cell Mito Stress Test was used to examine mitochondrial function and observed a similar effect in MCF-7 cells. Cells cultured and assayed at 5 % oxygen displayed a significant decrease in mitochondrial oxygen consumption over the normoxic control cells (Figure 5). The decrease in basal OCR is accompanied by a decrease in spare respiratory capacity and ATP-linked OCR. Together, these data suggest that mitochondrial activity is significantly limited following overnight exposure to 5 % O_2.

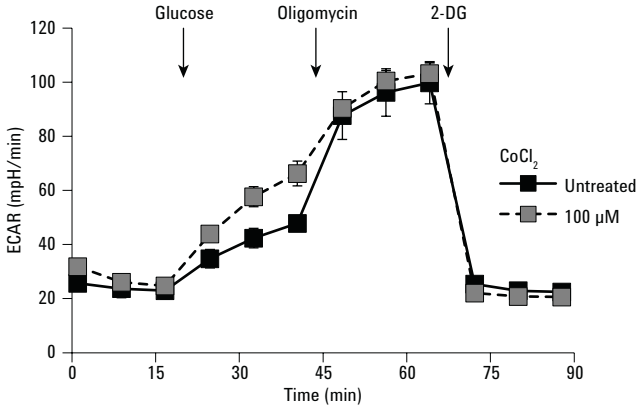

Figure 4. Treatment with cobalt chloride ($CoCl_2$) mimics the effect of exposure to low oxygen. MCF-7 cells were treated with 100 µM $CoCl_2$ for 16 hours. Stimulation of ECAR with injection of glucose (10 mM) was observed, similar to data shown in Figure 3. Data shown are means ± sd. n ≥ 7 per treatment group.

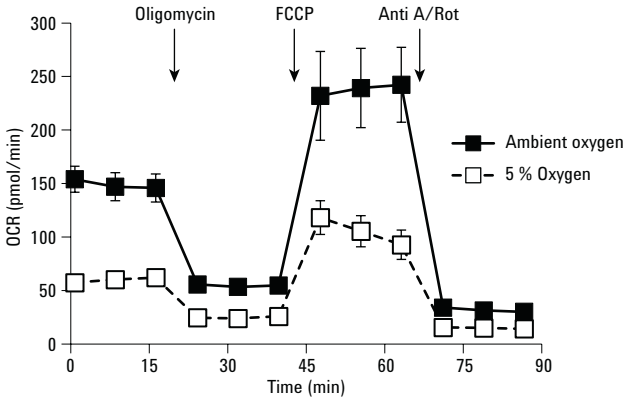

Figure 5. Basal OCR is depressed following overnight exposure to 5 %. In parallel experiments to the assays described above, the Agilent Seahorse XF Cell Mito Stress Test was also used on MCF-7 cells grown and assayed in either normoxia or 5 % O_2. Despite a lack of change in cell number with hypoxia-exposure, for this time period, basal oxygen consumption decreased significantly. Other parameters of mitochondrial function were also diminished, suggesting an overall decrease in mitochondrial amount or activity. Data shown are means ± sd. n ≥ 12 per treatment group.

Conclusions

Culturing cells under low-oxygen or hypoxic conditions will more closely simulate *in vivo* conditions and improve *in vitro* model systems. Moreover, accurately calculating OCR and ECAR in hypoxia-exposed cells will further the understanding of metabolic reprogramming in various pathologies.

This Application Note demonstrates that the XF and XFe Analyzers, in combination with a hypoxia chamber and software (that is, XF Hypoxia Rate Calculator or Hypoxia mode for Wave), is capable of measuring mitochondrial function and glycolysis in intact cells at 5 % oxygen levels.

Using the Agilent Seahorse XF Analyzer, and following the XF Hypoxia Assay outlined in the Protocol[4], we were able to observe a switch in the metabolic phenotype of hypoxia-exposed MCF-7 breast cancer cells. These cells appeared to be more glycolytic, and showed an overall decrease in mitochondrial function following prolonged exposure to 5 % O_2. These observations are in agreement with a recent study demonstrating a cancer cell-specific ability to up-regulate glycolysis in response to low oxygen[8].

Seahorse XF technology from Agilent Technologies provides a research tool to further explore metabolic analysis under low oxygen conditions to better understand the physiological and pathological effects. Moreover, the functionality demonstrated here is not limited to cancer research, but can have applications in stem cell biology and immunology, where the cells of interest have extraordinary metabolic flexibility, including an increased ability to respond to mitochondrial inhibition[9,10].

Assay Optimization Hints

Agilent recommends optimizing cell seeding density for each cell line to minimize the chance that cells will consume all the oxygen in the microchamber during measurement, creating an anoxic environment. In our experience, optimized cell densities were decreased by 1/3 to 1/2 compared to the densities commonly used at normoxia. Using these lower cell numbers may prevent an over-consumption of O_2 while maintaining consistent well-to-well consumption rates.

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